to all TLR agonists tested as compared to the medium control (FIG. **4**A). By contrast, pDC mainly upregulated HLA-DR, CD80 and CD86 upon R848 or R848+poly(I:C) stimulation and CD83 only upon CpG2216 stimulation (FIG. **4**A). A high proportion of in vitro derived XCR1$^+$ cDC expressed IFN-λ but not IFN-α, only upon TLR3 triggering, i.e. stimulation with poly(I:C) or R848+poly(I:C) (FIG. **4**B). They strongly expressed IL-12 only upon TLR8 triggering, i.e. stimulation with R848 or R848+poly(I:C) (FIG. **4**C). TNF was induced in these cells both by TLR3 and TLR8 triggering (FIG. **4**C). However, none of these cytokines were induced in XCR1$^+$ cDC stimulated through TLR9 (CpG) or TLR4 (LPS). In contrast, pDC from the same cultures expressed cytokines only upon TLR7 (R848) or TLR9 (CpG) triggering, with a high induction of IFN-α and TNF, a milder expression of IFN-λ but not expression of IL-12 (FIG. **4**B-C). Thus, the pDC and XCR1$^+$ cDC generated in vitro in our culture system faithfully mirror the known TLR responses of their in vivo counterparts.

[0141] In Vitro Generated pDC and XCR1$^+$ cDC Display Phenotypic Characteristics of their In Vivo Equivalents.

[0142] To better characterize our cultures, we analysed them for the surface expression of multiple classical DC subset markers. For a more unbiased analysis of our multi parameter flow cytometry data, we used the vi_SNE algorithm (Amir el-AD et al. viSNE enables visualization of high dimensional single-cell data and reveals phenotypic heterogeneity of leukemia. Nat Biotechnol. 2013 June; 31(6):545-52) which groups cell populations with similar expression patterns close to each other on the vi-SNE plots by taking into consideration all parameters analysed. When applied this algorithm to all live Lin$^-$ HLA-DR$^+$ cells (data not shown). We could thus identify a cluster of CD34(neg) CX3CR1(neg) BDCA2(low to neg) CD141(pos) CADM1 (pos) CLEC9A(pos) BTLA(pos) cells, and a cluster of CD34(neg) CX3CR1(low to neg) CADM1(neg) CLEC9A (neg) XCR1(neg) CD1c(neg) CD11c(neg) CD123(pos) BDCA2(pos) LILRA4(pos) BTLA(pos) cells, matching the phenotypes of blood XCR1$^+$ cDC and pDC respectively. Contrary to their blood counterparts, in vitro derived XCR1$^+$ cDC also expressed CD1c. However, it has been reported previously that XCR1$^+$ cDC derived in vitro from CB CD34$^+$ progenitors on MS5 stromal cells or isolated from Flt3L-injected human volunteers upregulate their CD1c expression (Breton et al. J Exp. Med. 2015). CD1c expression could thus possibly be upregulated due to the high concentrations of Flt3L in our culture system. The cluster of in vitro derived XCR1$^+$ cDC could be further divided into two subpopulations differing in their expression of CD123.

[0143] Single Cell RNA Sequencing Definitively Demonstrates the Homology Between In Vitro Derived XCR1$^+$ cDC and pDC and their In Vivo Counterparts and Unravels an Overlooked Heterogeneity within XCR1$^+$ cDC.

[0144] To further evaluate the degree of homology between the cells generated in vitro and their in vivo counterparts, and to assess possible heterogeneity of in vitro derived pDC and XCR1$^+$ cDC, we performed single cell RNA sequencing from cells cultured on OP9+OP9_DLL1 under FT7 conditions. All cells were sorted from a live Lin(neg) HLA-DR(pos) gate. pDC were sorted as CD141 (neg to low) CADM1(neg) BDCA2(pos) CD123(pos) cells. XCR1$^+$ cDC were sorted as CD141(pos) CADM1(pos) cells. In addition, as external references, we included two other putative DC populations identified in the culture by multidimensional flow cytometry analyses using the vi_SNE algorithm: CD141(low to neg) CADM1(neg) BDCA2(neg) CD123(neg) CD1c(pos) BTLA(pos) cells versus CADM1 (neg) BDCA2(neg) CD123(neg) CD1c(pos) BTLA(neg) cells. RNA isolation, downstream processing for sequencing and data bioinformatics analyses were performed based on a recently published method (Villani A C, et al. Single-cell RNA-seq reveals new types of human blood dendritic cells, monocytes, and progenitors. Science. 2017 Apr. 21; 356 (6335)). An unsupervised t-SNE analysis of the data identified 7 clusters of cells, based only on their gene expression profiles (data not shown). One cluster contained only, and the immense majority of, sorted pDC. Only 2 out of the 15 cells sorted as putative pDC did not fall in this cluster. The genes identified as specifically expressed to high levels in this cluster as compared to all other clusters encompassed many genes known to be specific of pDC (Robbins S H, et al. Novel insights into the relationships between dendritic cell subsets in human and mouse revealed by genome-wide expression profiling. Genome Biol. 2008 Jan. 24; 9(1):R17) (Crozat K, et al. Comparative genomics as a tool to reveal functional equivalences between human and mouse dendritic cell subsets. Immunol Rev. 2010 March; 234(1):177-98), including GZMB, PTCRA, NLRP7, SPIB, LILRA4, PACSIN1, CLEC4C, LILRB4, TCF4, IL3RA, NRP1, IRF7, EPHA2, TLR7, TEX2, CXXC5, PLAC8 and BLNK. Moreover, for this cell cluster as compared to all other ones, GeneSet Enrichment Analyses (GSEA) identified the transcriptomic fingerprints previously established for pDC as the gene signatures the most significantly enriched (Robbins et al. Genome Biol. 2008); (Carpentier S, et al. Comparative genomics analysis of mononuclear phagocyte subsets confirms homology between lymphoid tissue-resident and dermal XCR1(+) DCs in mouse and, human and distinguishes them from Langerhans cells. J Immunol Methods. 2016, May; 432:35-49); (See P, et al. Mapping the human DC lineage through the integration of high-dimensional techniques. Science. 2017 Jun. 9; 356(6342)). Two clusters contained only, and all of the, cells sorted as putative XCR1$^+$ cDC. The genes identified as specifically expressed to high levels in these clusters as compared to the other ones encompassed many genes known to be specific of XCR1$^+$ cDC (Robbins et al. Genome Biol. 2008), including CADM1, CLEC9A, IDO1, C1orf54, BATF3, SLAMF8, SNX22, CPNE3, GCSAM, THBD, WDFY4, IDO2 and CLNK. Moreover, for these 2 cell clusters as compared to all other ones, GeneSet Enrichment Analyses (GSEA) identified the transcriptomic fingerprints previously established for XCR1$^+$ cDC as the gene signatures the most significantly enriched (Robbins et al. Genome Biol. 2008; Carpentier et al. J Immunol Methods. 2016; Villani et al. Science. 2017; See et al. Science. 2017). Hence, Single cell RNA sequencing definitively demonstrated the homology between in vitro derived XCR1$^+$ cDC or pDC and their in vivo counterparts. In addition, this approach unravelled an overlooked heterogeneity within XCR1$^+$ cDC. Indeed, the two clusters identified for this cell type differed for the expression of cell cycle genes versus genes involved in the translation machinery and of CXCR4 versus XCR1. This suggested that our culture encompasses two differentiation states of XCR1$^+$ cDC: terminally differentiated cells expressing XCR1 versus their immediate precursors negative for XCR1 but expressing higher levels of CXCR4 and of cell cycle genes, which had not been identified before to the best of our knowledge. Flow cytometry analysis of in vitro derived CLEC9A$^+$ CADM1$^+$ cDC confirmed that these cells encompass two complementary populations based on their expression of XCR1 and CXCR4, and that this is also the case for their blood counterpart (data not shown).